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| 14. ABSTRACT Angiogenin is progressively upregulated in prostate cancer, in particular in androgen-independent diseases. The objective of this project is to explore the role angiogenin plays in the development of androgen-independent disease. In this reporting period, we have demonstrated that nuclear translocation of angiogenin is specific for prostate cancer cells and does not occur in normal prostate epithelial cells. Angiogenin is translocated to the nucleus of androgen-dependent cells (LNCaP) only when the cells are stimulated with androgen (DHT). But angiogenin is constitutively translocated to the nucleus of androgen-independent cells (PC-3, PC-3M, DU145). Angiogenin is required for DHT to stimulate rRNA transcription and cell proliferation of androgen-dependent cells. Overexpression of angiogenin enables androgen-independent growth of otherwise androgen-dependent prostate cancer cells in vitro and in vivo. Finally, we have shown that angiogenin binds to the promoter region of rDNA in vivo and stimulates rRNA transcription. | | | | | |
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Introduction

The goal of this study is to understand the molecular mechanism of angiogenin-mediated growth of androgen-independent growth of prostate cancer. This goal will be achieved by two specific aims. Specific aim 1 is to demonstrate the role of angiogenin in prostate cancer by characterizing the growth of angiogenin over-expressing LNCaP cells in castrated athymic mice and angiogenin under-expressing PC-3 cells in non-castrated mice. Specific aim 2 is to elucidate the mechanism by which angiogenin stimulates rRNA transcription. These two specific aims will be accomplished in 6 tasks.

In the previous year report, we had made substantial progress to task 1 (Months 1-9) and had completed task 2 (Months 10-18). In the current year report, we have completed task 1 and task 3 (Months 19-22) and have initiated task 4 (Months 23-27).

Body

Task 1: Task 1 is to characterize the growth of angiogenin over-expressing LNCaP cell in castrated athymic mice. In the last report we had shown that (1) Exogenous angiogenin stimulates proliferation of LNCaP cells but not that of PC-3, PC-3M, and DU145 cells, (2). Over-expression of angiogenin in LNCaP cells promotes androgen-independent proliferation *in vitro*. Now we have demonstrated that angiogenin overexpression enables LNCaP cell growth in castrated SCID mice (Fig. 1c).

1. Over-expression of angiogenin in LNCaP cells promotes androgen-independent proliferation and tumorigenesis

The angiogenin expression vector pCI-ANG that carries human angiogenin cDNA under the CMV promoter and the control vector pCI-Neo were transfected into LNCaP cells and stable transfectants expressing 6.6-times higher angiogenin were selected (Fig. 1a). Angiogenin over-expression stimulated LNCaP proliferation in the absence of androgen (Fig. 1b). To determine whether angiogenin over-expression promotes androgen-independent proliferation *in vivo*, these transfectants were inoculated into castrated SCID mice. Both the vector and angiogenin transfectants established tumors in uncastrated mice. However, only 1 of the 8 mice had palpable tumors in castrated mice inoculated with vector transfectants, whereas 7 of the 8 castrated mice developed tumors when inoculated with angiogenin transfectants (Fig. 1c). These results indicate that angiogenin over-expression enables LNCaP cells to proliferate in the absence of androgen both *in vitro* and *in vivo*.

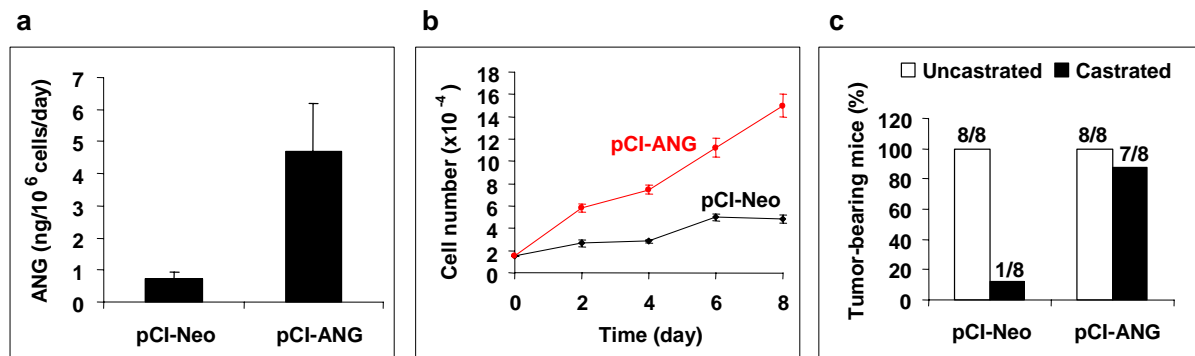


Fig. 1. Angiogenin over-expression promotes LNCaP proliferation *in vitro* and *in vivo*. (a) Angiogenin secretion levels as determined by ELISA. (b) *In vitro* proliferation in the absence of androgen. The vector control and angiogenin transfectants were seeded at 1×10^4 cells per 35-mm dish and cultured in RPMI 1640 + 10% FBS for 24h. The cells were washed with serum-free medium 3 times and switched to steroid-free medium (phenol red-free RPMI 1640 + 10% charcoal/dextran-stripped FBS). (c) Xenograft growth in castrated nude mice. A mixture of 70 μ l cell suspension (1×10^6 cells) and 30 μ l Matrigel was injected s.c. per mouse (8 per group). The mice were castrated or sham-operated at the same time. Tumors were inspected and measured twice a week and the animals were sacrificed after 8 weeks of observation.

Task 3: Task 3 is to define the *in vivo* binding site of angiogenin to ribosomal RNA. We have completed all the experiments proposed in task 3. Chromatin immunoprecipitation (ChIP) experiments have demonstrated that angiogenin binds to three regions on the rDNA promoter. We have also demonstrated that angiogenin stimulates rRNA transcription and is required for DHT-mediated rRNA transcription and cell proliferation.

1. Angiogenin binds to the promoter region of ribosomal DNA (rDNA) and stimulates rRNA transcription

Our previous *in vitro* study has identified three angiogenin binding DNA elements (ABE) in the promoter region of rDNA¹. ABE has been shown to have angiogenin-dependent promoter activity in a luciferase reporter assay¹. However, it remained unknown whether angiogenin binds to ABE *in vivo* and whether this binding is responsible for rRNA transcription. We therefore studied *in vivo* binding of ANG to the promoter region of rDNA by chromatin immunoprecipitation (ChIP).

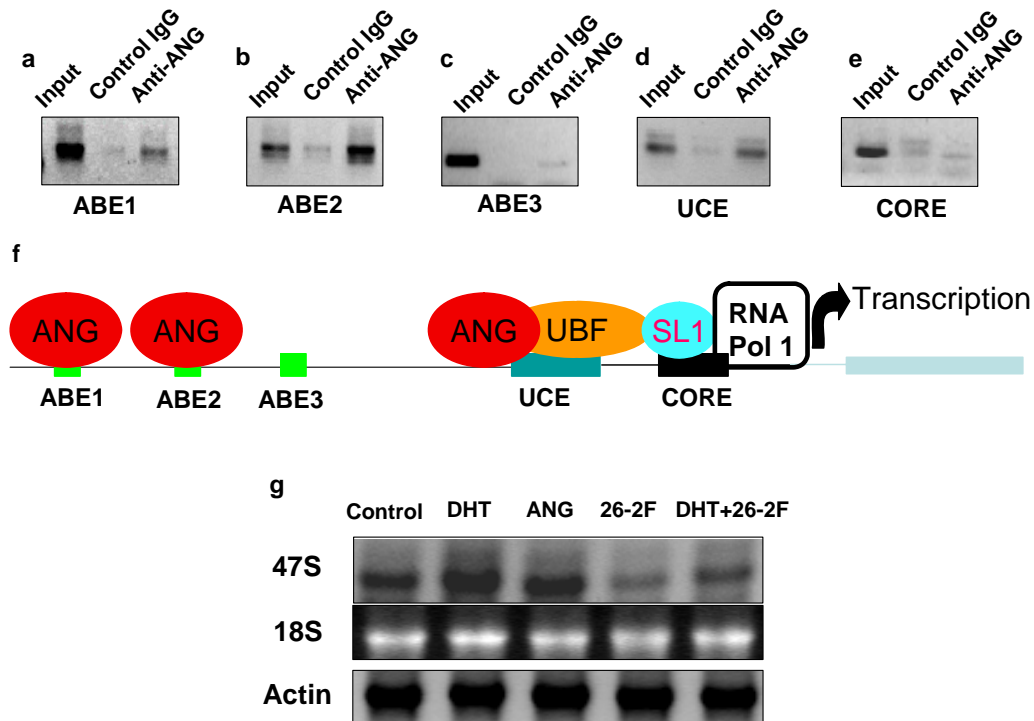


Fig. 2. Angiogenin binds to the promoter region of rDNA and stimulates rRNA transcription in LNCaP cells. (a-e) ChIP analyses of angiogenin binding to ABE1 (a), ABE2 (b), ABE3 (c), UCE (d), and CORE (e) region of the rDNA promoter. PCR primers for the sites of ABE1, ABE2, ABE3, UCE, and CORE were designed using MacVector software and have been confirmed to amplify specific amplicons from input LNCaP genomic DNA. The input control in each panel contains 0.2% of the total DNA. (f) A schematic view of the rDNA with ABE shown in green and angiogenin protein shown in red. (g) Northern blotting analysis of 47S rRNA precursor. LNCaP cells were cultured in charcoal/dextran-stripped FBS and incubated with DHT (1 nM), angiogenin (0.1 µg/ml), 26-2F (60 µg/ml) or a mixture of DHT and 26-2F for 2 h. The level of 47S rRNA was analyzed by Northern blotting with a probe specific to the initiation site sequence of the rRNA precursor. EB staining of 18S rRNA and Northern blotting of actin mRNA was used as the loading controls.

Fig. 2 shows that angiogenin binds to ABE1, ABE2, and to the upstream control element (UCE) where the essential transcription factor UCE binding factor (UBF) binds. Fig. 2f is a schematic illustration of angiogenin binding to the promoter region of rDNA. rRNA transcription in LNCaP cells is stimulated by exogenous angiogenin and inhibited by anti-angiogenin IgG 26-2F as shown by Northern blotting analysis (Fig. 2g). Moreover, 26-2F inhibited androgen dihydrotestosterone (DHT)-induced rRNA transcription, indicating that DHT-induced rRNA transcription is mediated by angiogenin.

3. Angiogenin is constitutively translocated to the nucleus of androgen-independent prostate cancer cells

These results, together with the results in the previous year report, suggest that nuclear angiogenin undergoes nuclear translocation where it binds to the promoter region of ribosomal DNA, stimulates rRNA transcription, and thereby drives cell proliferation. Nuclear translocation is thus a prerequisite for the nuclear function of angiogenin. We therefore examined nuclear translocation of ANG in androgen-dependent and androgen-independent prostate cancer cells in the presence or absence of androgen.

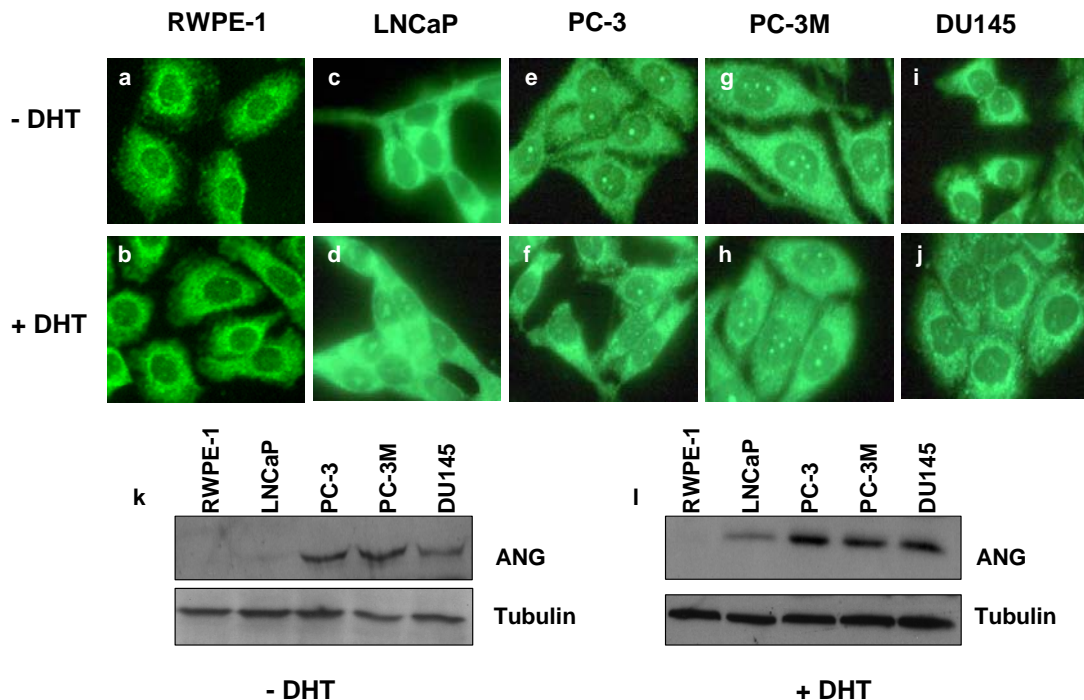


Fig. 3. Nuclear translocation of angiogenin in prostate cancer cells. RWPE-1, LNCaP, PC-3, PC-3M, and DU145 cells were cultured in their respective media supplemented with 10% FBS for two days. FBS was then replaced with charcoal/dextran-stripped serum (steroid-depleted medium) and the cells were cultured in the absence (**a,c,e,g,i**) or presence (**b,d,f,h,j**) of DHT (10 nM) for another 2 days. (**a-j**) Immunofluorescence of angiogenin detected with anti-angiogenin monoclonal antibody 26-2F (50 µg/ml) and Alexa 488-labeled goat F(ab')₂ anti-mouse IgG (1:100 dilution). Nucleolar stainings of angiogenin were indicated by arrows. (**k,l**) Nuclear proteins were extracted from the cells cultured in the absence (**k**) and presence (**l**) of DHT and analyzed by Western blotting (150 µg per lane) with an anti-angiogenin polyclonal antibody (R112).

No nuclear angiogenin was detectable by immunofluorescence in RWPE-1 normal prostate epithelial cells either in the absence or presence of DHT (Fig. 3a,b). LNCaP cells express a promiscuous gain-of-function mutant androgen receptor (AR)² and are able to respond to the physiological level of androgens. Nuclear angiogenin was detected in LNCaP cells only when the cells were stimulated with DHT (Fig. 1c,d). PC-3, PC-3M, and DU145 cells are deficient in AR and grow in the absence of androgens³. Angiogenin is constitutively translocated to the nucleoli of these cells both in the absence and in the presence of DHT (Fig. 3e-j). Western blotting analysis with an anti-human angiogenin polyclonal antibody R113 (Fig. 3k,l) showed that in PC-3, PC-3M and DU145 cells, comparable amounts of angiogenin protein were detected when equal amounts of nuclear protein, extracted from cells cultured in the absence (Fig. 3k) or presence (Fig. 3l) of androgen, were applied. However, in LNCaP cells, Angiogenin was detectable only in the nuclear protein extracted from DHT-stimulated cells (Fig. 3l). No angiogenin protein was detected by Western blotting in the nuclear protein extracted from RWPE-1 cells cultured under both conditions. Thus, nuclear translocation of angiogenin is specific for prostate cancer cells and does not occur in normal prostate epithelial cells. Furthermore, there is a correlation between nuclear translocation of angiogenin and the growth status of the cancer cells.

Task4: Task 4 is to determine the effect of angiogenin on ribosomal promoter occupancy by SL1, an essential transcription factor for rRNA. We have initiated this task and have prepared AAV-angiogenin siRNA. This siRNA will be used to knockdown angiogenin expression in LNCaP cells. Together with the addition of exogenous angiogenin and anti-angiogenin antibody, we will be able to manipulate angiogenin levels in LNCaP cells and determine the resultant changes in SL1 binding to rDNA promoter by ChIP assays.

Key Research Accomplishment

- Demonstrated that angiogenin overexpression enable LNCaP cells to establish tumor in castrated mice. Provided in vivo evidence that angiogenin plays a role in the transition of prostate cancer to androgen independence.
- Demonstrated that angiogenin binds to the promoter region of ribosomal DNA. Determined the in vivo binding sites and proposed a mechanism by which angiogenin stimulates rRNA transcription.
- Demonstrated that angiogenin is required for androgen to induce rRNA transcription in androgen-dependent prostate cancer cells.
- Determined the differential nuclear translocation pattern of angiogenin in normal prostate epithelial cells, androgen-dependent and androgen-independent prostate cancer cells.

Reportable outcomes

- 1) An abstract entitled “Angiogenin-mediated rRNA transcription plays a role in androgen-independent prostate cancer cells” was presented as a poster in the 2007 Annual meeting of the American Association for Cancer Research. Abstract number #4155.
- 2) An abstract entitled “Role of angiogenin in progression to androgen-independent prostate cancer” was presented as an oral presentation in the 2007 DOD Innovative Minds in Prostate Cancer Today (IMPACT) meeting. Abstract number # S11-4.

Conclusion

In this reporting period, we have shown that angiogenin binds to ABE in the promoter region of rDNA and stimulates rRNA transcription. We have also shown that angiogenin is constitutively in the nucleus of androgen-independent cells, suggest that upregulation and constitutive nuclear translocation of angiogenin will result in a constant supply of rRNA, which may contribute to the development of androgen independency through the so-called “bypass” AR pathway⁴. We have shown that rRNA transcription in LNCaP cells in response to DHT stimulation is inhibited by anti-angiogenin IgG, indicating that AR-stimulated rRNA transcription is mediated by angiogenin. Consistently, angiogenin has been found to undergo nuclear translocation in LNCaP cells upon DHT stimulation. Moreover, anti-angiogenin IgG abolishes DHT-induced LNCaP cell proliferation in a dose-dependent manner. These results suggest that angiogenin is required for DHT to stimulate cell proliferation. Finally, exogenous angiogenin can compensate for the loss of androgen-AR signaling axis and ectopic expression of angiogenin enables LNCaP cells to proliferate in the absence of androgen and to establish tumors in castrated mice. Together, these results clearly demonstrate that angiogenin plays an essential role in AR-dependent cell proliferation and that upregulation of angiogenin may contribute to the development of androgen independence.

The research is on track. No changes or modifications are foreseen for the next 12 months.

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Appendices

Two meeting abstracts

[Proc Amer Assoc Cancer Res, Volume 47, 2006]

Cellular and Molecular Biology 55: Extracellular Signaling Abstract #4155

Angiogenin-mediated rRNA transcription plays a role in androgen-independent growth of prostate cancer cells

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Progression to androgen-independent disease remains the major barrier to effective treatment of prostate cancer. The ability of prostate cancer to acquire an androgen-independent phenotype has been associated with changes in androgen receptor (AR). Metastatic hormone refractory prostate cancer has a heterogeneous morphology and phenotype among which AR-deficient cells exist indicating that alternative pathways can be invoked that are capable of bypassing the AR completely. We hypothesize that angiogenin-mediated rRNA transcription is one such mechanism. Angiogenin is an angiogenic ribonuclease whose expression is upregulated in human prostate cancer tissues and cells. Its concentration is progressively increased in the plasma of prostate cancer patients as the disease progresses from androgen-dependent to androgen-independent status. Here we examined the effect of angiogenin on androgen-independent growth of prostate cancer cells both in vitro and in vivo. The results show that exogenous angiogenin stimulates the proliferation of androgen-responsive LNCaP cells in the absence of androgens and other steroid hormones. However, exogenous angiogenin has no effect on the proliferation of androgen-independent prostate cancer cell lines including PC-3, PC-3M, and DU-145. Knocking down the expression of endogenous angiogenin in PC-3 cells by siRNA resulted in a decrease in rRNA transcription, ribosome biogenesis, and cell proliferation. Soft agar assay shows that both the colony number and size decreased in angiogenin siRNA transfectants. exogenous angiogenin was able to compensate the loss of endogenous angiogenin mediated by siRNA in the colony formation assay. Moreover, tumor therapy experiments show that neamine and neomycin, aminoglycosides that block nuclear translocation of angiogenin, inhibit xenografic growth of PC-3 cells in athymic mice. These results demonstrate that angiogenin stimulates androgen-independent growth of prostate cancer cells and suggest that angiogenin may play a role in the progression of prostate cancer to androgen independence. This work was supported by NIH grant CA105241 and DOD grant PC050976 to GFH .

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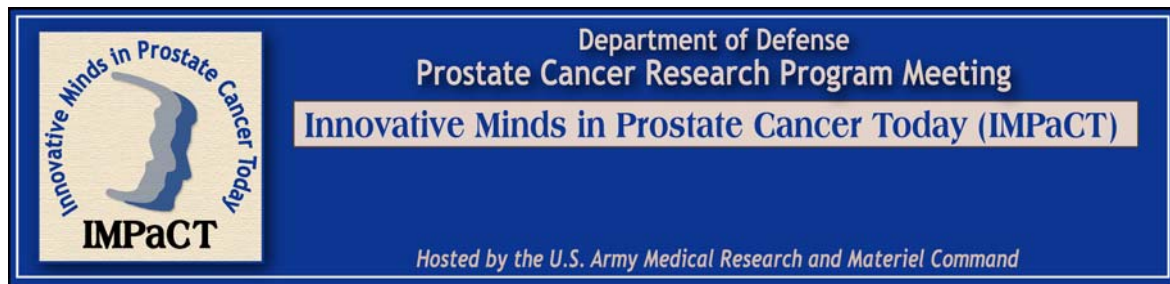
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Guo-Fu Hu, Ph.D.
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Dear Dr. Hu,

Congratulations! On behalf of the Prostate Cancer Research Program (PCRP) and the Technical Planning Committee for the Innovative Minds in Prostate Cancer Today (IMPACT) meeting, I am pleased to inform you that your abstract "A Novel Function of Angiogenin in Androgen-Independent Prostate Cancer" has been accepted for a 10-minute oral presentation in the session "Angiogenesis" from 11:15 a.m. to 12:45 p.m. on Thursday, September 6.

You will receive an email invitation directing you how to reserve a hotel room and request travel assistance in about 1 month. At that time, you will also receive more detailed information about your session.

We look forward to your participation in the inaugural IMPACT meeting.

Sincerely,

Theresa J. Miller
Program Chair
Innovative Minds in Prostate Cancer Today

Role of angiogenin in progression to androgen-independent prostate cancer

Norie Yoshioka, Hiroko Kishikawa and Guo-fu Hu

Department of Pathology, Harvard Medical School, Boston, Massachusetts

Angiogenin is an angiogenic ribonuclease that is progressively upregulated in prostate cancer. We have recently shown that in addition to its well established role in mediating tumor angiogenesis, angiogenin is also directly involved in prostate cancer cell proliferation (Yoshioka et al., PNAS, 103, 14519-14524, 2006). Angiogenin is translocated to the nucleus of prostate cancer cells where it stimulates the transcription of ribosomal RNA (rRNA), a primary target of the androgen-androgen receptor signal axis. The goal of this study is to explore the role angiogenin may play in androgen-independent progression of prostate cancer, and to develop novel therapeutic means based on inhibition of the function of angiogenin.

By means of immunofluorescence, we have studied the nuclear translocation patterns of angiogenin in normal prostate epithelial cells and in androgen-dependent and androgen-independent prostate cancer cells. We have also studied the effect of angiogenin on prostate cancer cell proliferation in the presence and absence of androgen in vitro and in vivo.

We have found that angiogenin is constitutively translocated to the nucleus of androgen-independent prostate cancer cells (PC-3, PC-3M, and DU145) both in the absence and presence of androgen. However, nuclear translocation of angiogenin in androgen-dependent LNCaP cells occurs only when the cells are stimulated with androgen. Exogenously added angiogenin has no effect on PC-3 cells but is able to stimulate LNCaP cell proliferation in the absence of androgen and other steroid hormones. Angiogenin over-expression also stimulates LNCaP cell proliferation in the absence of androgen. On the other hand, knocking down endogenous angiogenin in PC-3 cells inhibits both anchorage dependent and independent cell proliferation, as well as xenograft growth in athymic mice. Moreover, anti-angiogenin monoclonal antibody inhibits DHT-induced LNCaP cell proliferation. We have also found that neomycin, an aminoglycoside antibiotic, blocks nuclear translocation of angiogenin in endothelial cells and in prostate cancer cells. Thus, neomycin inhibits PC-3 cell tumor growth in athymic mice accompanied by a decrease in both cancer cell proliferation and angiogenesis.

Mouse angiogenin has been reported to be the most upregulated gene in the prostatic intraepithelial neoplasia (PIN) tissue of the murine prostate-specific Akt transgenic (MPAKT) mice. We have now found that upregulation of angiogenin in prostate epithelial cells occurs much earlier (4 weeks) than the PIN phenotype is developed, suggesting that upregulation of angiogenin might be a cause rather than a consequence of PIN development.

These results suggest that constitutive nuclear translocation of angiogenin in prostate cancer cells is a contributing factor for the development of androgen independence and that angiogenin-stimulated rRNA transcription is a novel therapeutic target for this disease.